Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 7391–7395, August 1991
Cell Biology

Localization of pre-messenger RNA at discrete nuclear sites

(rhodamine-labeled pre-mRNA/nuclear microinjection/small nuclear ribonucleoproteins/spliceosomes)

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Communicated by Joseph G. Gall, May 31, 1991 (received for review May 2, 1991)

ABSTRACT **We have studied the nuclear localization of rhodamine-labeled pre-mRNA after microinjection into nuclei of cultured rat kidney epithelial cells. Intranuclear localization of the injected RNA was followed in the living cells by fluorescence microscopy and digital image processing. Injected human β-globin pre-mRNA became localized in 30–60 discrete nuclear sites that were coincident with loci defined by monoclonal antibodies against small nuclear ribonucleoproteins (Sm) or another spliceosome component (SC-35) in parallel immunocytochemical studies on the same nuclei. Similar patterns of nuclear localization were observed with a rat proenkephalin pre-mRNA. Nuclear microinjection of an intron-lacking β-globin RNA, a splicing-defective β-globin mutant pre-mRNA, or an antisense β-globin pre-mRNA did not result in localization at discrete sites. These results indicate that pre-mRNA binds preferentially to nuclear Sm and SC-35 antibody-reactive sites *in vivo* and that the binding requires intron sequences.**

Pre-messenger RNAs transcribed from intron-containing genes undergo splicing before exiting from the nucleus. Components of the splicing apparatus have recently been localized in discrete nuclear sites by immunocytochemistry of fixed mammalian somatic cells (1, 2), and potentially analogous structures have been described in dispersed nuclei from amphibian oocytes (3, 4). Immunocytochemical experiments at the electron microscopic level have revealed that the splicing-related small nuclear ribonucleoproteins (snRNPs) are associated with perichromatin fibrils, interchromatin granules, and coiled bodies, which are structures that are known to contain heterogeneous nuclear RNA and pre-mRNA (5, 6). snRNPs have also been shown to be colocalized with pre-mRNA transcripts on insect polytene chromosomes (7, 8). However, the nuclear sites at which splicing actually takes place have not been defined.

In the present investigation, our objective was to determine whether exogenous pre-mRNA has an affinity for a particular element of nuclear structure. We have developed a system in which rhodamine-labeled pre-mRNA is introduced into the nucleus of normal rat kidney (NRK) epithelial cells, and its intranuclear distribution is followed by fluorescence microscopy of the living cells.

MATERIALS AND METHODS

Rhodamine Labeling of RNA. A human β-globin pre-mRNA containing exon 1, intron 1, and 209 nucleotides of exon 2 (9) was transcribed from *Bam*HI-cut plasmid SP64-HβΔ6 with SP6 RNA polymerase in the presence of 500 μM ATP, CTP, GTP, and UTP and 50 μM 5-(3-aminoallyl)-UTP (Sigma) (10). The RNA was recovered by ethanol precipitation after phenol and chloroform extraction and was coupled to tetramethylrhodamine-6-isothiocyanate (Molecular Probes) as described (11). The coupling reactions contained 25 mM sodium bicarbonate buffer (pH 9.0) and 16% (vol/vol) dimethylformamide. The rhodamine–RNA coupling reaction was carried out for 8–12 hr in the dark at 20°C in a tube mounted on a rocking platform. After the reaction, the RNA was purified by gel filtration through either Sephadex G-25 or Bio-Gel P-60 (with comparable results). RNA from the peak column fractions was concentrated by ethanol precipitation and then further purified by electrophoresis in an 8% polyacrylamide gel containing 7 M urea.

To evaluate the efficiency of rhodamine conjugation, a preparation of HβA RNA was transcribed in a sufficient amount for an A_{260} reading. After rhodamine conjugation, the rhodamine–RNA was purified by Bio-Gel P-60 gel filtration, ethanol precipitation, and electrophoresis. After elution from the gel and ethanol precipitation, the RNA concentration was measured as A_{260} and the amount of coupled dye was measured by spectrofluorimetry at an excitation wavelength of 536 nm. The average value for three independent RNA preparations was 7.9 molecules of rhodamine per molecule of RNA. The rhodamine coupling efficiency was calculated by considering that the 497-nucleotide HβA RNA contains 128 uridines (12) and by assuming that 1/11th of these uridines, or 11.6, carry aminoallyl groups (because transcription was done with 500 μM UTP and 50 μM aminoallyl-UTP). Thus, the rhodamine coupling efficiency can be estimated to be 7.9/11.6 or 68%.

Nuclear Microinjection of RNA. All RNA samples for microinjection were purified by gel filtration and ethanol precipitation, followed by preparative gel electrophoresis, elution, and ethanol precipitation. RNA was dissolved in 5 mM Tris acetate buffer (pH 6.95) at a concentration of ≈50 μg/ml for microinjection. NRK cells were grown in F12K medium (Hazelton Research Products, Lenexa, KS) with 10% fetal calf serum (Hazelton). NRK cells are a permanent line, having an aneuploid karyotype but retaining density-dependent inhibition of motility (13). Microinjection experiments were carried out in growing, subconfluent cells at 36.5°C. After heating at 65°C for 2 min, the RNA was drawn into a glass needle and injected into the nucleus of a selected cell with a Leitz micromanipulator and a custom-designed electronic air pressure regulator. Typically, 0.01 pl of solution (no more than 0.5 fg of RNA) was injected per nucleus. After injection, the recipient cell was monitored by fluorescence microscopy (excitation λ, 546 nm) and digital image processing with an ISIT camera (Dage–MTI, Michigan City, IN), as described in detail (14, 15). Image acquisition and processing were performed with a 4D/20 workstation (Silicon Graphics, Mountain View, CA) in conjunction with series no. 150 image processing boards (Imaging Technology, Woburn, MA). For the detection of extremely faint structures, 300 video frames were summed into a 16-bit frame buffer, and the background obtained by summing 300 dark images was subtracted. The resulting images were repeatedly divided by

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Abbreviation: snRNP, small nuclear ribonucleoprotein.

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2 until the maximal pixel value became <256 (and thus could be displayed as 8-bit images) and were then stored as computer files on a hard disk. Photographs were taken directly off the monitor screen using Kodak Tri-X film developed in Diafine (Acufine, Chicago).

Immunocytochemistry. Rhodamine-coupled pre-mRNA was injected into the nucleus as described above. After the desired period of time, the cells were permeabilized and fixed (2) and then incubated with either Sm monoclonal antibody (16) or a monoclonal antibody to a non-snRNP spliceosome component, SC-35 (2). The sites of antibody binding were detected with fluorescein-conjugated goat anti-mouse IgG. The fluorescence of rhodamine-labeled RNA was observed with a Zeiss 487715 filter set, which consists of a BP546/12 excitation filter, an FT580 dichroic mirror, and an LP590 barrier filter. The fluorescence of fluorescein-labeled secondary antibody was observed with a Zeiss 487717 filter set, which consists of a BP485/20 excitation filter, an FT510 dichroic mirror, and a BP520-560 barrier filter. No crossover between fluorescein and rhodamine fluorescence was detectable.

RESULTS AND DISCUSSION

A human β-globin pre-mRNA containing the first intron (hereafter referred to as HβA) was transcribed with SP6 RNA polymerase in the presence of 5-(3-aminoallyl)-UTP and was subsequently conjugated with rhodamine via the aminoallyl groups (average of ≈8 molecules of rhodamine per molecule of RNA; see *Materials and Methods*). The labeled RNA could be visualized by its fluorescence after purification and gel electrophoresis (Fig. 1 *A* and *B*). As shown in Fig. 1*C*, the rhodamine-labeled HβA RNA underwent the first step of splicing in a HeLa nuclear extract to approximately the same extent as HβA RNA that had not been dye coupled.

After microinjection of ≈2000 molecules of rhodamine–HβA RNA into each nucleus of NRK epithelial cells, the intranuclear distribution was followed by fluorescence microscopy and digital image processing. Fig. 2 illustrates the results of a typical experiment. At 5 min after microinjection, the RNA was distributed throughout the nucleoplasm (Fig. 2*a*). Over the next 13–63 min, the RNAs became progressively localized at discrete multiple loci within the nucleo-

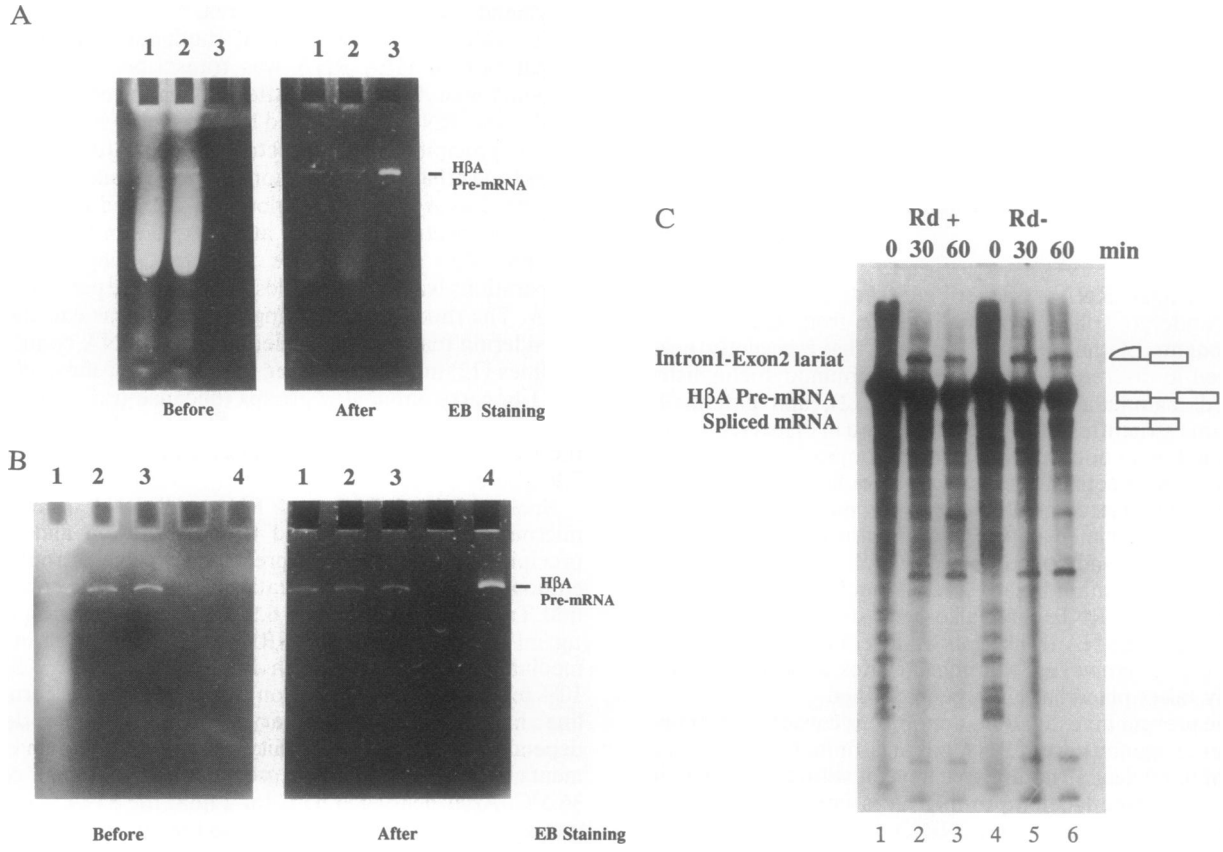

FIG. 1. Rhodamine labeling and *in vitro* splicing competence of human β-globin pre-mRNA. (*A*) Lanes: 1, HβA RNA transcribed without aminoallyl-UTP and not conjugated to rhodamine; 2, HβA RNA transcribed with aminoallyl-UTP followed by rhodamine coupling; 3, HβA RNA transcribed with aminoallyl-UTP but not subjected to rhodamine coupling. (*Left*) Unstained; excitation with 254-nm light. Note the band in lane 2. (*Right*) Same gel as shown on the left, but after ethidium bromide (EB) staining. (Three times more total HβA RNA was loaded on lane 3 than on lanes 1 and 2.) (*B*) Purification of rhodamine-coupled RNA. HβA RNA was transcribed and coupled to rhodamine as described in *A*. Lanes: 1, the RNA–rhodamine was loaded directly on the gel; 2, rhodamine–RNA after Bio-Gel P-60 gel filtration and ethanol precipitation; 3, rhodamine–RNA shown in lane 2 after an additional gel electrophoresis purification step; 4, HβA RNA transcribed in the absence of aminoallyl-UTP and not subjected to rhodamine coupling. (*Left*) Excitation with 254-nm light (note the labeled RNA bands in lanes 1–3). (*Right*) Same gel as shown on the left, but after ethidium bromide staining. (Three times more total HβA was loaded on lane 4 than on lanes 1–3.) (*C*) *In vitro* splicing of rhodamine-labeled β-globin pre-mRNA. HβA RNA was transcribed with SP6 RNA polymerase in the presence of 50 μCi of [α-^{32}P]CTP (1 Ci = 37 GBq) (reaction vol, 50 μl), 500 μM ATP and UTP, 50 μM GTP and CTP, and 50 μM aminoallyl-UTP. The RNA was recovered, labeled with rhodamine, and purified as described above. A parallel HβA transcription reaction was carried out with [α-^{32}P]CTP but without aminoallyl-UTP and without subsequent rhodamine coupling of the RNA. The RNAs were incubated in a HeLa cell nuclear extract as described (9, 17) for the indicated times, followed by phenol extraction, ethanol precipitation, electrophoresis in an 8% polyacrylamide/7 M urea gel, and autoradiography. Lanes: 1–3, rhodamine-labeled (Rd+) HβA RNA; 4–6, HβA RNA not labeled with rhodamine (Rd−). The electrophoretic positions of the lariat intermediate, the pre-mRNA and the spliced product are indicated at the left of lane 1. Densitometry of the film revealed that the percentage of input HβA detected as lariat intermediate or spliced product was not appreciably different between the rhodamine-coupled (≈8 dye molecules per RNA) and noncoupled RNAs.

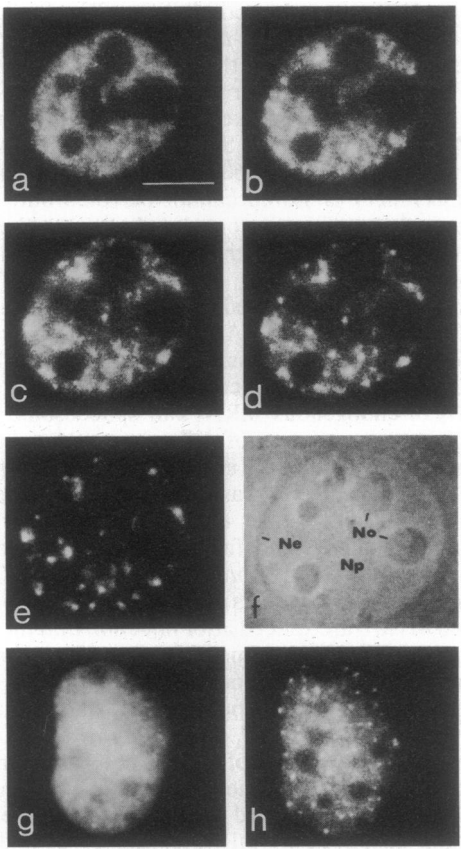

FIG. 2. pre-mRNA localization after nuclear injection. (*a–f*) Human β-globin pre-mRNA (HβA) was transcribed and rhodamine-labeled as described in Fig. 1. (*a*) At 5 min after injection. (*b–e*) At 18, 27, 36, and 68 min after injection. (*f*) Phase-contrast microscopy (at 68 min). No, nucleoli; Np, nucleoplasm; Ne, nuclear envelope. (*g* and *h*) Nuclear injection of rat proenkephalin pre-mRNA. A rat proenkephalin pre-mRNA (733 nucleotides) containing intron A_S and exons I_S and II_S was generated by T3 RNA polymerase transcription of a previously described plasmid (18), kindly provided by Daniel Kilpatrick (Worcester Foundation). The RNA was rhodamine labeled, purified, and injected as detailed above. (*g*) At 5 min after nuclear injection. (*h*) Same nucleus 39 min after injection. (Bar = 10 μm.)

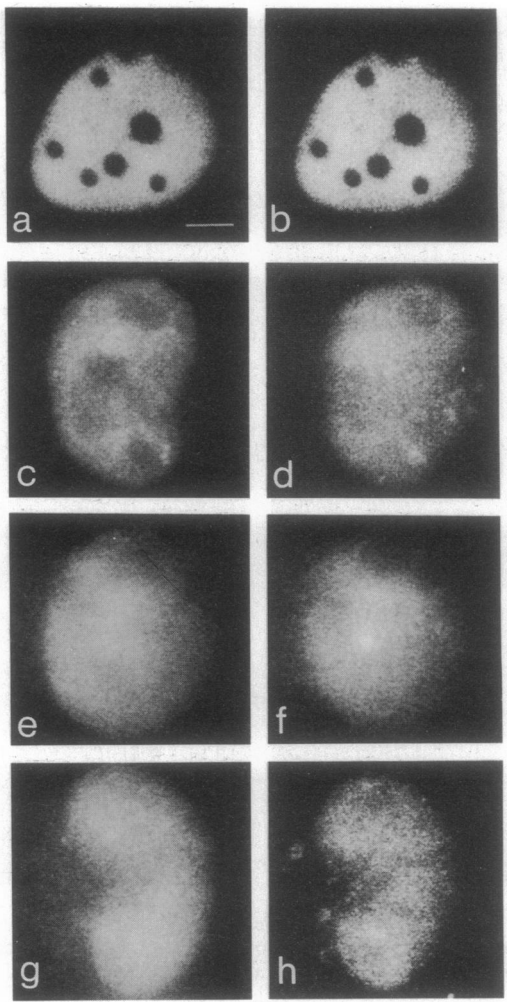

FIG. 3. Nuclear injection of rhodamine–dextran and splicing-defective control RNAs. (*a* and *b*) Rhodamine-labeled dextran (average M_r, 71,200) (Sigma) was microinjected into a cell nucleus and monitored as usual. (*a*) At 5 min after injection. (*b*) At 35 min after injection. (*c–h*) Rhodamine-labeled, splicing-defective RNAs. An antisense β-globin pre-mRNA (514 nucleotides long, essentially antisense to HβA; see ref. 19), an intron-lacking β-globin RNA, and a mutant β-globin pre-mRNA lacking the polypyrimidine tract and 3′ splice site were transcribed from the plasmids pT7HβΔ6 (19), SP62 Hβ-IVS1,2 (20), and ΔPyAG (21), respectively. Rhodamine labeling and microinjection were carried out as described in Fig. 1 and 2. (*c* and *d*) Antisense β-globin RNA, 7 and 34 min after injection, respectively. (*e* and *f*) Intron-lacking β-globin RNA, 9 and 23 min after injection, respectively. (*g* and *h*) ΔPyAG β-globin RNA, 5 and 35 min after injection, respectively. (Bar = 5 μm.)

plasm (Fig. 2 *b–e*). The RNAs did not localize in nucleoli. Similar patterns of nuclear localization were observed after injection of a rhodamine-labeled rat proenkephalin pre-mRNA (Fig. 2*h*) or an adenovirus pre-mRNA (data not shown).

Several control experiments demonstrated that the localization shown in Fig. 2 is specific for unspliced pre-mRNA. Injection of rhodamine-conjugated dextran (average M_r, 71,200) resulted in rapid dispersion throughout the nucleoplasm with no localization at discrete sites (Fig. 3 *a* and *b*). Injection of rhodamine-labeled antisense β-globin pre-mRNA (Fig. 3 *c* and *d*) or rhodamine-labeled β-globin RNA containing exons 1 and 2, but lacking intron 1 (Fig. 3 *e* and *f*) also resulted in diffuse intranuclear patterns. Moreover, a rhodamine-labeled, splicing-defective mutant β-globin pre-mRNA containing a deletion of the intron polypyrimidine tract and the 3′ splice site did not accumulate in discrete loci (Fig. 3 *g* and *h*).

Over the course of many injections of β-globin pre-mRNA, ≈65% of the nuclei displayed the characteristic 30–60 sites of localization. The remaining ≈35% of the injected nuclei displayed a diffuse pattern, although with a degree of localization in some cases. In contrast, in no case was localization observed with the splicing-defective control RNAs.

The typical pattern of nuclear pre-mRNA localization (Fig. 2) is very similar to previously reported patterns of immunoreactivity with spliceosome component antibodies (1, 2, 16, 22–24). To determine whether these sites are one and the same, we injected rhodamine-labeled β-globin pre-mRNA into the nucleus, allowed the RNA to localize, and then fixed the cell and carried out immunocytochemistry experiments with either Sm monoclonal antibody, which is specific for proteins common to several splicing-related snRNPs (16), or a monoclonal antibody, α-SC-35, which is specific for a non-snRNP spliceosome (2). As shown in Fig. 4, the sites of Sm and SC-35 antibody reactivity corresponded very closely to the pattern of injected pre-mRNA localization observed in

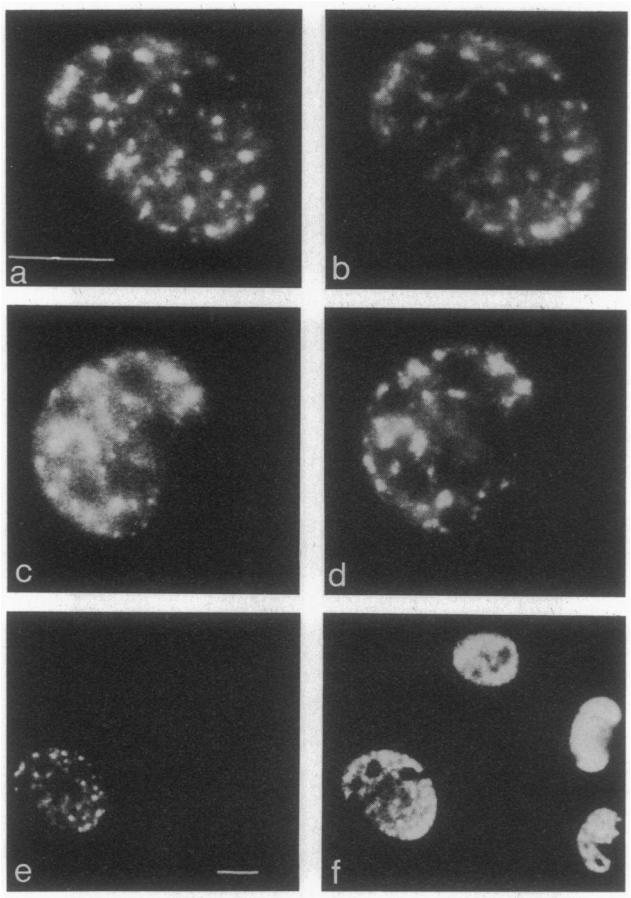

FIG. 4. Pre-mRNA microinjection and parallel immunocytochemical localization of spliceosome components. Rhodamine-coupled HβA RNA was injected and monitored as described in Fig. 2. At 30 min postinjection, the cells were permeabilized and fixed (2) and then incubated with either Sm monoclonal antibody or monoclonal antibody to SC-35. (*a*) RNA. (*b*) Sm antibody. (*c*) RNA. (*d*) SC-35 antibody. (*e*) RNA. (*f*) Sm antibody (photographic exposure deliberately overset). The field shown in *e* and *f* includes the injected nucleus shown in *a* and *b*; note that only the one injected nucleus of the four in *f* shows RNA fluorescence (*e*). Magnifications in *a–d* are calibrated on the bar in *a*; magnifications in *e* and *f* are calibrated on the bar in *e*. (Bars = 10 μm.)

the same nuclei. Thus, living cells procure injected pre-mRNA into nuclear sites that contain endogenous snRNPs and spliceosomal proteins. These results also demonstrate, importantly, that fixation and subsequent antibody staining do not alter the *in vivo* pattern of pre-mRNA localization.

We noted that the intensity of fluorescence at the discrete nuclear sites decreased 30 min postinjection. However, it is difficult to know whether this represents mRNA export. Since we inject ≈2000 molecules of pre-mRNA, the 30–60 sites of localization could each maximally contain 33–66 molecules. (It is to be emphasized that these sites cannot be seen in the fluorescence microscope with the unaided eye but require image intensification for visualization.) If some of the introduced nuclear RNA were exported or released to the cytoplasm during our typical 5- to 75-min period of observation, it would likely be below the detection threshold of our system.

In these experiments, we do not know whether the injected pre-mRNA undergoes splicing, either before or after its arrival at the observed sites of localization. However, the lack of localization observed with an intron-lacking β-globin cDNA transcript and with a splicing-defective mutant pre-mRNA demonstrates that intron sequences are required. Our results also demonstrate that a 3′ poly(A) tail is not required for pre-mRNA localization at the observed sites. It is conceivable that our results are a visualization of active splicing centers, but there are other possibilities. For example, it is possible that endogenous pre-mRNA does not interact with these sites but that they nonetheless contain elements (e.g., snRNPs) that have affinity for the injected pre-mRNA. This scenario is rendered less likely by the coincidence of a non-snRNP splicing cofactor, SC-35 (2), with these sites, but the possibility that the 30–60 sites are regions at which splicing machinery is stored cannot be ruled out. Another possibility is that excised introns are shuttled to these sites, perhaps for debranching after the second step of splicing carried out elsewhere.

It has recently been reported that both U2 RNA and a U2 snRNP-associated factor, U2AF, are confined to one to five nuclear foci (25, 26). It is possible that the nuclear localizations of U2 RNA or U2AF as compared to that of exogenous pre-mRNA or endogenous snRNPs or spliceosome proteins might reflect the restriction of different components of the splicing machinery to distinct spatial domains, depending on their use or storage. In any case, neither these experiments (25, 26) nor the present ones elucidate the nuclear sites of active mRNA splicing, and further studies are clearly required to resolve this important issue.

We note that several studies have shown that pre-mRNAs transcribed *in vitro* are spliced after injection into the nucleus of frog oocytes (27–30), demonstrating that transcription and splicing are not obligatorily coupled in this system. Comparable experiments injecting pre-mRNAs into somatic cell nuclei have not been reported, due mainly to the difficulty of injecting a sufficient number of cells to detect splicing of the recovered RNA. However, the fact that oligo(dT)-cellulose- or poly(U)-Sepharose-selected nuclear RNA from vertebrate cells contains unspliced pre-mRNAs (refs. 31–33 and numerous subsequent studies) demonstrates that, at least for some transcripts, splicing occurs after poly(A) addition—i.e., after transcription. This consideration does not obviate the possibility that splicing also occurs on some nascent pre-mRNAs in vertebrate somatic cells, as has been visualized in *Drosophila* preblastoderm embryos (34, 35). In any case, our results are compatible with these earlier findings in demonstrating that intranuclear movement of pre-mRNA to, and localization at, the observed nuclear sites is not obligatorily coupled to transcription. We suspect that in those cases in which splicing follows the completion of transcription, the pre-mRNA may move only a short distance from its site of transcription before it associates with the first splicing center it encounters, a possibility compatible with previous results (1, 7).

The methods and results we have presented here open a potential path to resolving several aspects of RNA traffic in living cells. These include further definition of pre-mRNA sequences required for localization at these nuclear sites, the intranuclear localizations of RNA polymerase III-transcribed RNA precursors, and the dynamics with which small nuclear RNA precursors exit the nucleus and return (36–38). In addition, the intracellular traffic of antisense DNA, antisense RNA, or ribozymes can be investigated in living cells by the direct observation methods we have presented here.

Note Added in Proof. We have recently learned of experiments by K. Carter, K. Taneja, and J. Lawrence (University of Massachusetts Medical School) demonstrating that poly(A) is concentrated in the numerous discrete nuclear sites that react with Sm antibody (personal communication).

We thank Sudhir Agrawal and Jin-Yan Tang for key suggestions on coupling rhodamine to RNA, James Hammarback for advice on immunocytochemistry, Harvey Florman for helping us perform the spectrofluorimetry, the members of the Pederson laboratory for critical comments on the manuscript, and Carol Tuttle for able word

processing. We also thank Xiang-Dong Fu and Tom Maniatis (Harvard University) for the SC-35 hybridoma, and Charles Janeway (Yale University) for the Y-12 (Sm) hybridoma. This work was supported by National Institutes of Health Grants GM-32476-07 to Y.-L.W. and GM-21595-16 to T.P., a core grant from the National Cancer Institute, and funds from the G. Harold and Leila Y. Mathers Foundation. J.W. and L.-G.C. are graduate students in the joint PhD program of Worcester Polytechnic Institute and the Worcester Foundation for Experimental Biology.

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